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Control of Memory CD4 T Cell Activation: MHC Class II Molecules on APCs and CD4 Ligation Inhibit Memory but Not Naive CD4 T Cells

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Summary

Memory or antigen-experienced CD4 T cells differ from naive CD4 T cells both phenotypically by cell surface marker expression, and functionally by their dissimilar pattern of cytokine secretion and activation requirements through their T cell receptor (TCR). We show here that activation of memory CD4 T cells (CD45RB^{lo} subset), but not naive CD4 T cells (CD45RB^{hi} subset), is inhibited by MHC class II molecules on antigen-presenting cells and by CD4 ligation. We propose that the selective negative signal in memory cells is a direct result of the differences in signaling via CD4 and CD3, exemplified in the disparate pattern of tyrosine-phosphorylated proteins visible after activation of the two subsets. In vivo, this inhibitory signal may serve to prevent irrelevant interactions between memory CD4 T cells and bystander MHC class II$^+$ cells, and may also be responsible for the defective functioning of memory CD4 T cells in AIDS.

Introduction

A memory or recall immune response protects against reexposure to a particular pathogen and differs from a primary response in rapidity, magnitude, and duration. Memory is manifested by the generation of antigen-specific T cells that differ from naive T cells: quantitatively, by their increased precursor frequency due to previous expansion during the primary response, and qualitatively, by their production of a diverse array of cytokines and by their longevity (Mackay, 1991; Gray, 1993; Bradley et al., 1993). Memory CD4 T cells can be phenotypically distinguished from naive CD4 T cells based on the expression of various cell surface markers, including distinct isoforms of the CD45 glycoprotein (Smith et al., 1986; Sanders et al., 1988; Salmon et al., 1989; Lee et al., 1990; Dianzani et al., 1990), the adhesion molecule CD44, or Pgp-1 (Sanders et al. 1988; Budd et al., 1987), and the homing receptor MEL-14 (Lee and Vitetta, 1991; Bradley et al., 1992). Isolation of memory and naive CD4 T cells based on these surface markers has enabled characterization of functional differences between these two CD4 T cell subsets.

Studies of CD45-defined subsets in human (Byrne et al., 1988; Horgan et al., 1990; Sanders et al. 1989) and in mouse (Luqman and Bottomly, 1992) have revealed that memory CD4 T cells differ from naive CD4 T cells in their activation requirements for stimulation through the T cell receptor (TCR)–CD3 complex. As we have previously reported (Luqman and Bottomly, 1992), mouse memory CD4 T cells (CD45RB^{lo} subset) proliferate in response to anti-CD3 immobilized on plastic surfaces, while naive cells (CD45RB^{hi} subset) do not. However, naive CD4 T cells can be induced to proliferate if anti-CD3 is presented by Fc receptor (FcR)–bearing antigen-presenting cells (APCs) from normal spleen, suggesting that naive cells have a greater requirement for costimulatory signals than memory cells.

During the course of these studies, we made the paradoxical observation that although naive CD4 T cells were activated in the presence of anti-CD3 and T-depleted splenic APC, memory CD4 T cells failed to respond to this stimulus. The fact that memory CD4 T cells could respond to anti-CD3 immobilized on plastic or cross-linked by FcR-bearing fibroblasts suggested that splenic APCs were delivering an inhibitory signal selectively to the memory CD4 T cells. As it is well known that CD4 cross-linking can inhibit CD4 T cell responses to anti-CD3 and other mitogens (Bank and Chess, 1985; Tite et al., 1986; Newell et al., 1990; Rosoff et al., 1987; Wassmer et al., 1985; Janeway et al., 1988), we have examined the role of the CD4 ligand, major histocompatibility complex (MHC) class II, and of the CD4 molecule itself in this inhibitory effect.

We find that inhibition of memory CD4 T cell activation by spleen APCs plus anti-CD3 requires expression of MHC class II molecules on the cell presenting anti-CD3. Thus, anti-CD3 presented by FcR$^+$/MHC class II positive (II$^+$) APC prevents activation of memory CD4 T cells, whereas anti-CD3 presented by FcR$^+$ APC that lack MHC class II molecules (II^0) stimulates memory CD4 T cells to proliferate and produce effector cytokines such as interferon-γ (IFNγ) and interleukin-4 (IL-4). Moreover, ligation of CD4 on memory CD4 T cells reproduces the inhibitory effect of MHC class II$^+$ APC but does not inhibit naive CD4 T cell activation, further demonstrating that signals that impede memory CD4 T cell activation can be delivered through the CD4 molecule. A molecular basis for this differential activation is manifested by the dissimilar pattern of tyrosine-phosphorylated proteins that appear after CD4 or CD3 cross-linking in the two subsets, suggesting that CD4 and CD3 are coupled to different signaling pathways in the two cell types.

We propose that the inhibitory signal delivered selectively by CD4 on CD45RB^{lo} CD4 T cells may serve to prevent these memory cells from bystander activation by MHC class II–bearing cells in the absence of a TCR-mediated signal. These results also suggest that activation

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Table 1. Summary of Differential Activation Requirements of CD45RB^{lo} and CD45RB^{hi} Cells

Stimulus[a]	CD45RB^{lo}	CD45RB^{hi}
Immobilized anti-CD3	++	–
Soluble anti-CD3	–	–
Soluble anti-CD3 plus CHO/FcγRII cells[b]	+++	–
Soluble anti-CD3 plus T-depleted splenocytes[c]	–	+++

[a] All results summarized from Luqman and Bottomly, 1992, unless otherwise indicated.
[b] Cells are described (Luqman and Bottomly, 1992).
[c] Results presented here.

of memory CD4 T cells may be stringently controlled in ways that differ from the regulation of naive CD4 T cell activation.

Results

Mouse CD4 T cells can be separated on the basis of CD45RB isoform expression using the 16A monoclonal antibody (MAb) to exon 5–encoded determinants of CD45. CD4 T cells separated in this way have been shown to represent memory (CD45RB^{lo}) and naive (CD45RB^{hi}) CD4 T cell subsets (Lee et al., 1990; Dianzani et al., 1990; Bottomly et al., 1989). As previously demonstrated (Luqman and Bottomly, 1992), CD45RB^{lo} CD4 T cells comprising the memory subset, and CD45RB^{hi} CD4 T cells, comprising the naive subset, exhibited disparate reactivities to anti-CD3 presented in different contexts. These data are summarized in Table 1. In response to plastic-immobilized anti-CD3, known to be a potent stimulus for T cells (Samelson et al., 1987), CD45RB^{lo} CD4 T cells proliferated well, whereas CD45RB^{hi} CD4 T cells failed to proliferate. This differential activation was duplicated in response to soluble anti-CD3 presented by FcR-bearing CHO fibroblast transfectants. The only stimulus that induced proliferation of CD45RB^{hi} CD4 T cells was soluble anti-CD3 presented by T-depleted splenocytes as APC. Such "professional" APC cross-link anti-CD3 bound to TCR/CD3 on T cells via surface FcR, and also provide costimulatory activity (Liu and Janeway, 1992). Thus, memory CD4 T cells defined by low expression of CD45RB could be activated solely by cross-linking TCR/CD3, whereas naive CD4 T cells, defined by high expression of CD45RB, required both TCR/CD3 cross-linking and an additional stimulus provided by a professional APC.

CD45RB^{lo} CD4 T Cells Respond to Immobilized Anti-CD3 but Fail to Respond to Anti-CD3 plus Splenic APC by Proliferation and Cytokine Synthesis

Further analysis of naive and memory CD4 T cell activation yielded the unexpected finding that memory CD4 T cells did not respond to anti-CD3 presented by T-depleted spleen cells as APC. In these experiments, naive and memory CD4 T cells were prepared from resting CD4 T cells and separated using anti-CD45RB antibodies into

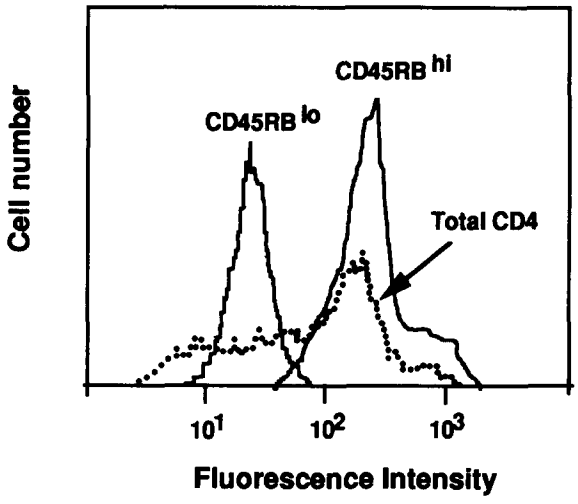

Figure 1. CD45RB Surface Expression of Splenic CD4$^+$ T Cells Before Sorting and CD45RB^{lo} and CD45RB^{hi} Subsets After Magnetic Bead Sorting on the MACS

CD4$^+$ cells were stained with C363.16A–biotin, followed by streptavidin magnetic beads and fluorescein isothiocyanate–avidin, separated on the MACS, and analyzed on the FACScan.

CD45RB^{hi} (naive) and CD45RB^{lo} (memory cells) (Figure 1). The CD45RB^{lo} subset also expressed low levels of MEL-14 and high levels of Pgp-1 (data not shown), consistent with a memory cell phenotype. These cells were then assayed for their ability to respond to anti-CD3 presented by T-depleted splenocytes (Figure 2A). CD45RB^{hi} CD4 T cells proliferated vigorously to anti-CD3 presented by APC, consistent with previous findings (Luqman and Bottomly, 1992), whereas CD45RB^{lo} CD4 T cells failed to proliferate in response to this stimulus. CD45RB^{lo} CD4 T cells proliferated well in response to immobilized anti-CD3 as well as anti-CD3 presented by FcR$^+$ CHO cells (Luqman and Bottomly, 1992; data not shown), demonstrating that these cells respond to TCR/CD3 cross-linking. The selective inhibition of CD45RB^{lo} CD4 T cells by anti-CD3 and splenic APC was demonstrated at a broad range of antibody concentrations and at different timepoints (data not shown).

To determine whether other features of memory CD4 T cell responses, such as effector cytokine release, were also inhibited in the presence of spleen APC, production of IL-4 and IFNγ by anti-CD3-activated CD45RB^{lo} CD4 T cells was measured. As demonstrated in Figure 2B, CD45RB^{lo} CD4 T cells produced IL-4 and IFNγ in response to activation by immobilized anti-CD3, but produced very low levels of these cytokines in response to anti-CD3 and T-depleted splenocytes. Thus, cross-linking of TCR/CD3 on the surface of CD45RB^{lo} CD4 T cells by FcR$^+$ splenic APC inhibits both proliferation and effector cytokine release. This inhibition is selective for the memory (CD45RB^{lo}) subset and does not affect the response of naive CD4 T cells expressing high levels of CD45RB.

These results indicate that T-depleted spleen cells deliver an inhibitory signal that blocks T cell proliferation and cytokine production by CD45RB^{lo} CD4 T cells activated

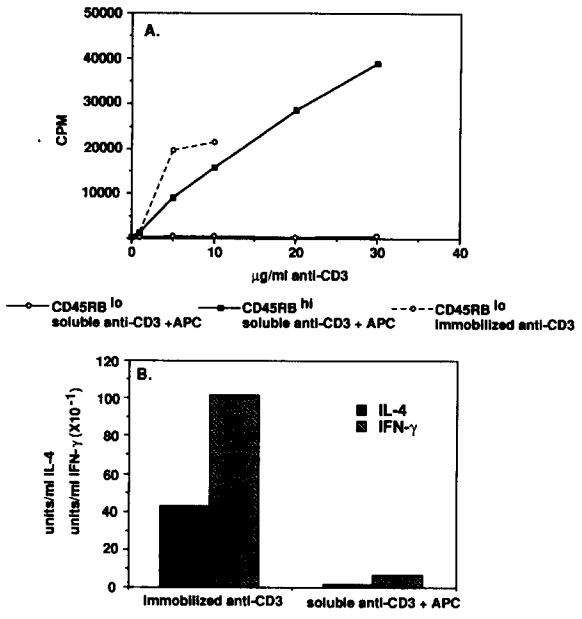

Figure 2. The CD45RB^{lo} Memory Subset Fails to Respond to Stimulation by Anti-CD3 plus APC

(A) Proliferative response of CD45RB^{lo} versus CD45RB^{hi} cells to anti-CD3(29B) plus T-depleted splenocytes compared with proliferation of CD45RB^{lo} cells to plastic immobilized anti-CD3. Proliferation was measured after 4 days as described in Experimental Procedures.

(B) Cytokine profile of CD45RB^{lo} cells following treatment with immobilized anti-CD3 or anti-CD3 and APC. IL-4 and IFNγ were measured by bioassay (see Experimental Procedures) with 1 U defined as half-maximal response.

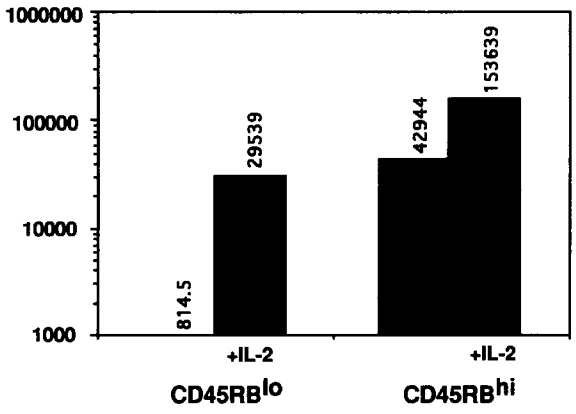

Figure 3. The Failure of Memory CD4 T Cells to Respond to Anti-CD3 and Class II$^+$ APC Can Be Overcome by Providing IL-2

Proliferative responses of CD45RB^{lo} and CD45RB^{hi} CD4 T cells to 10 μg/ml anti-CD3 and MHC class II$^+$ APC ± 50 U/ml mouse recombinant IL-2. Background proliferation with IL-2 alone was <2000 cpm.

by FcR cross-linked anti-CD3. The experiments that follow seek to define the nature of the molecules that mediate the negative effects on CD45RB^{lo} CD4 T cells.

CD45RB^{lo} CD4 T Cells Are Signaled by Anti-CD3 plus Splenic APC as Shown by Induction of Proliferation to Exogenous IL-2

Two possible explanations can account for the failure of CD45RB^{lo} CD4 T cells to respond to anti-CD3 presented by T-depleted spleen cells; either this form of presentation kills the responding T cells or it fails to signal them. Although examination of CD45RB^{lo} CD4 T cells by trypan blue staining 48 hr after incubation with anti-CD3 and APC revealed no overt cell death (data not shown), both of these possibilities can be ruled out by adding exogenous IL-2 to the cultures. As shown in Figure 3, stimulation with anti-CD3 and T-depleted spleen cells induces CD45RB^{lo} CD4 T cells to proliferate in response to IL-2. IL-2 also enhances the proliferative response of naive CD45RB^{hi} CD4 T cells to anti-CD3 and T-depleted spleen cells. These results indicate that memory CD4 T cells remain viable and are signaled in the presence of anti-CD3 and splenic APC, but do not proliferate unless provided with exogenous IL-2. However, the CD45RB^{lo} cells do not appear anergic, because following treatment with anti-CD3 and splenic APC, they are still able to respond to immobilized anti-CD3 (data not shown).

MHC Class II Expression by FcR$^+$ Cells Inhibits Proliferation of and Effector Cytokine Secretion by Memory but not Naive CD4 T Cells Induced by Anti-CD3

Because memory CD4 cells could be activated by immobilized anti-CD3 or anti-CD3 presented by FcR-bearing fibroblasts but not by FcR-bearing T-depleted splenic APC, it seemed likely that splenic APC surface structures not expressed by fibroblasts were inhibiting memory CD4 T cell activation. It has been shown previously that cross-linking of CD4 can deliver an inhibitory signal to CD4 T cells activated by anti-receptor antibodies or mitogenic lectins (Bank and Chess, 1985; Tite et al., 1986; Wassmer et al., 1985; Haque et al. 1987). The natural ligand of CD4 is MHC class II, which is known to bind and cross-link CD4 (Doyle and Strominger, 1987). Since FcR$^+$ cells in T-depleted spleen are MHC class II$^+$ and inhibit CD45RB^{lo} CD4 T cell responses to anti-CD3, whereas MHC class II^0 FcR$^+$ fibroblasts support such responses, we asked whether MHC class II is the crucial molecule required for inhibition of memory CD45RB^{lo} CD4 T cell responses to anti-CD3.

To address this question, the full-length cDNA encoding murine FcγRIIb2 (Ravetch et al., 1986) was transfected into MHC class II^0 and into I-A^d-expressing MHC class II$^+$ fibroblasts (König et al., 1992). The resultant transfectants, L/FcR$^+$/II^0 and L/FcR$^+$/II$^+$, were paraformaldehyde fixed to prevent fibroblast proliferation and tested for their ability to activate anti-CD3-treated CD45RB^{lo} CD4 T cells. As shown in Figure 4A, both L/FcR$^+$/II^0 and L/FcR$^+$/II$^+$ express comparable amounts of FcR, whereas only L/FcR$^+$/II$^+$ expresses I-A^d. When anti-CD3 was presented to CD45RB^{lo} CD4 T cells by these two transfectants, only the MHC class II$^-$ (L/FcR$^+$/II^0) fibroblasts induced proliferation (Figure 4B). By contrast, MHC class II$^+$ fibroblasts (L/FcR$^+$/II$^+$) failed to induce proliferation even at high anti-CD3 concentrations. Thus, the presence of MHC class II on a nonprofessional APC inhibits memory CD4 T cell activation.

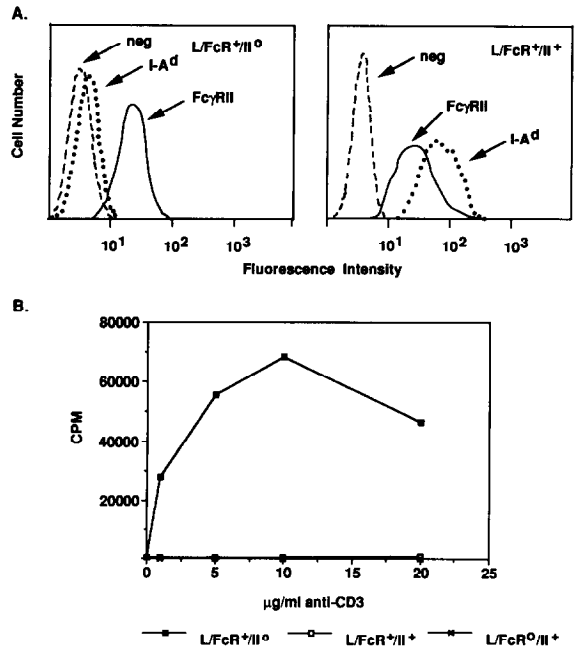

Figure 4. Memory CD4 T Cell Response to FcR$^+$ Fibroblasts Is Regulated by MHC Class II

(A) FACS profile showing FcγR and MHC class II surface expression by L cell transfectants as assessed by staining with MAbs 2.4G2 and 212.A1, respectively.

(B) Proliferative response of CD45RB^{lo} cells to soluble anti-CD3 presented by L/FcR$^+$/II^0, L/FcR$^+$/II$^+$, and L/FcR^0/II$^+$ transfectants.

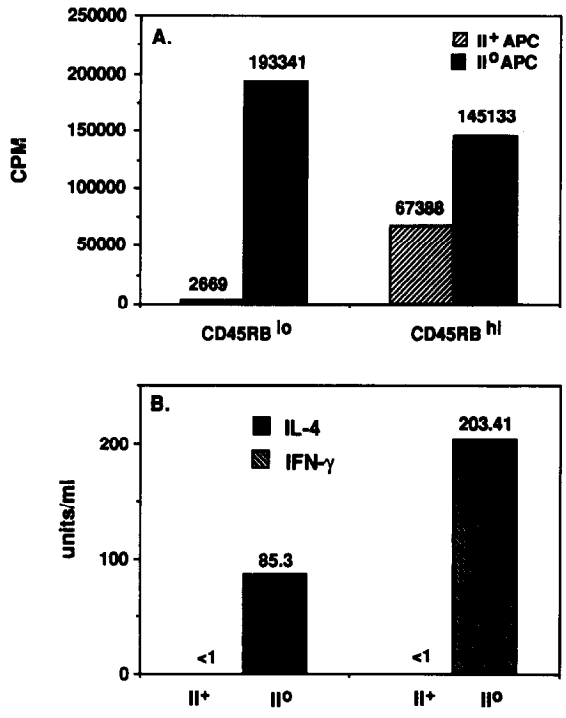

Figure 5. Memory CD4 T Cell Responsiveness Is Controlled by Expression of MHC Class II on the Splenic APC

(A) Proliferative response of CD45RB^{lo} CD4 T cells to 5 μg/ml anti-CD3 plus class II$^+$ APC versus class II$^-$ APC (II^0) isolated from MHC class II knockout mice. The following cpm were obtained when no anti-CD3 was added: CD45RB^{lo} plus II$^+$APC, 380; CD45RB^{lo} plus II^{0}APC, 343; CD45RB^{hi} plus II$^+$APC, 143; CD45RB^{hi} plus II^{0}APC, 196.

(B) Cytokine production by CD45RB^{lo} cells in response to soluble anti-CD3 plus class II$^+$ APC or class II^0 APC.

As our original data describing memory CD4 T cell unresponsiveness involved presentation of anti-CD3 by splenic APC and not by fibroblasts, the ability of T-depleted splenocytes obtained from mice defective for MHC class II expression to present anti-CD3 was also analyzed. As seen in Figure 5A, anti-CD3 presented by either MHC class II$^+$ or MHC class II^0 splenic APC strongly stimulated proliferation of naive CD4 T cells, although proliferative responses were somewhat stronger when MHC class II^0 splenic APC were used. By contrast, anti-CD3 presented by MHC class II$^+$ splenic APC did not induce proliferation of CD45RB^{lo} CD4 T cells, whereas anti-CD3 presented by MHC class II^0 T-depleted spleen cells induced excellent proliferative responses. Moreover, production of the effector cytokines IL-4 and IFNγ by memory CD45RB^{lo} CD4 T cells occurred only when anti-CD3 was presented by MHC class II^0 cells and not when it was presented by MHC class II$^+$ cells (Figure 5B).

Both MHC class II^0 and MHC class II$^+$ APC express equivalent levels of FcγRII, as assessed by staining with MAb 2.4G2 and anti-CD3-treated memory cells formed comparable numbers of rosettes with MHC class II$^+$ and MHC class II^0 APC (data not shown), eliminating the possibility that MHC class II^0 APCs are capable of more extensive cross-linking via their Fc receptors. Moreover, proliferation induced by the MHC class II^0 APCs themselves can be ruled out because no proliferation was observed when CD45RB^{lo} or CD45RB^{hi} cells were incubated with MHC class II^0 APC alone (Figure 5, legend), and anti-CD3-induced proliferation by MHC class II^0 APC was completely inhibited by the anti-FcγRII MAb, 2.4G2 (see Figure 7).

Thus, the presence of MHC class II molecules on an FcγR-bearing cell inhibits activation of CD45RB^{lo} memory CD4 T cells stimulated by soluble anti-CD3 while not significantly affecting identical stimulation of naive CD45RB^{hi} CD4 T cell responses.

Inhibitory Effects of MHC Class II$^+$ APCs Are Not Observed In Trans

Given the ability of CD45RB^{lo} cells to respond to anti-CD3 presented by MHC class II^0 APC, but not to antibody presented by MHC class II$^+$ APC, we asked whether contact with MHC class II$^+$ APC distinct from anti-CD3 cross-linking was sufficient to shut off memory cell activation. Thus, if activating and inhibitory signals are uncoupled, and therefore not delivered by the same accessory cell, can CD45RB^{lo} cells then be activated?

To address this question, we used two approaches. First, we treated CD45RB^{lo} cells with anti-CD3 presented by FcR$^+$/MHC class II^0 splenocytes plus either FcR^0/MHC class II$^+$ fibroblasts (L/FcR^0/II$^+$) to determine whether this two-cell combination would exhibit the same inhibitory effects as the FcR$^+$/II$^+$ presenting cells (see Figure 4B; Fig-

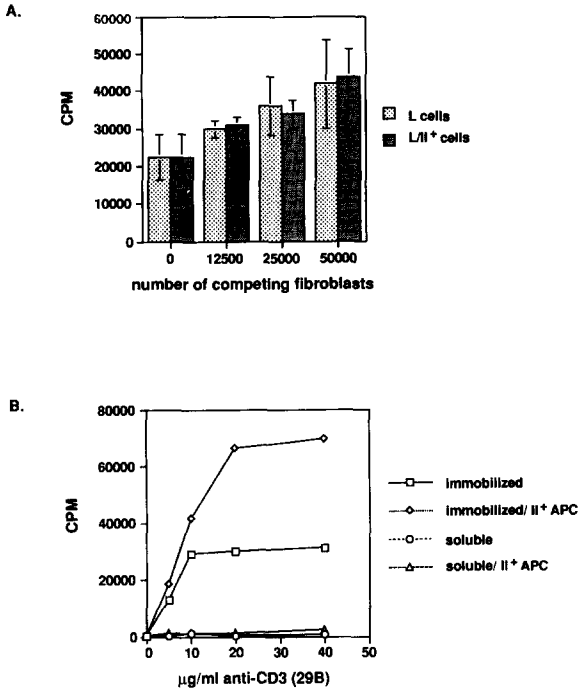

Figure 6. Inhibition of CD45RB^{lo} Activation by MHC Class II Cannot Occur In Trans

(A) CD45RB^{lo} cells (50,000 per well) were incubated with 10 μg/ml of anti-CD3 plus MHC class II^{0} T-depleted splenocytes (75,000 per well) plus increasing amounts of paraformaldehyde-fixed L cells or L cells expressing MHC class II molecules (L/II^{+}) and proliferation was measured after 4 days.

(B) CD45RB^{lo} cells (25,000 per well) were incubated with anti-CD3 in immobilized or soluble form ± MHC class II^{+} T-depleted splenocytes (75,000 per well) and proliferation was assessed after 4 days.

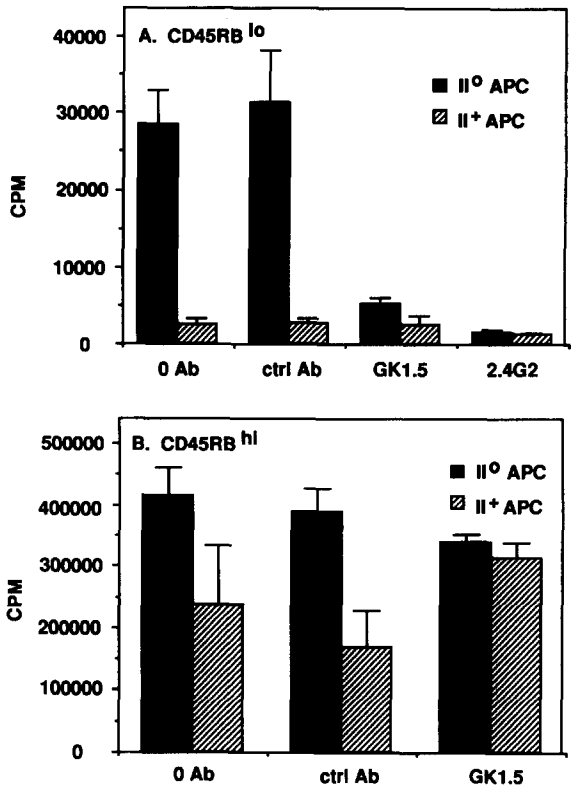

Figure 7. Cross-Linking CD4 Inhibits CD45RB^{lo} CD4 T Cell Proliferation Induced by MHC Class II^{0} APC but Does Not Block CD45RB^{hi} Proliferation to MHC Class II^{+} or MHC Class II^{0} APC

(A) CD45RB^{lo} CD4 T cells were treated with 10 μg/ml anti-CD3 and T-depleted splenocytes from BALB/c (II^{+} APC) or MHC class II knockout mice (II^{0} APC) ± 10 μg/ml of an isotype-matched control antibody (TIB 210, anti-CD8), anti-CD4 (GK1.5), or anti-FcRII (2.4G2), and proliferation was assessed as described above.

(B) CD45RB^{hi} CD4 T cells in the same experiment were treated as in (A) ± TIB 210 control antibody or GK1.5. Addition of as much as 20 μg/ml of GK1.5 did not inhibit CD45RB^{hi} CD4 T proliferation to II^{+} APC or II^{0} APC (data not shown).

ure 6A). As shown in Figure 6A, presentation of anti-CD3 by MHC class II^{0} splenocytes plus L/FcR^{0}/II^{+} stimulated CD45RB^{lo} cells to proliferate to the same extent as with L/FcR^{+}/II^{0} plus L cells added as controls, indicating that the addition of MHC class II^{+} cells in trans was not sufficient to inhibit memory cell proliferation to an activating stimulus.

To determine whether the same mechanism was operative with MHC class II^{+} splenic APC, we used a second approach and asked whether contact with splenic MHC class II^{+} APC could inhibit CD45RB^{lo} activation by immobilized anti-CD3. As shown in Figure 6B, immobilized anti-CD3 alone stimulated CD45RB^{lo} proliferation, whereas immobilized anti-CD3 plus splenic APC augmented this proliferation 2-fold. Again, soluble anti-CD3 plus the same ratio of MHC class II^{+} APC failed to activate CD45RB^{lo} cells. The activation induced by immobilized anti-CD3 plus APC was not due to FcR-mediated cross-linking of anti-CD3 bound to T cells, because this proliferation was not inhibited by an anti-FcR antibody 2.4G2, at a concentration that completely blocked CD45RB^{hi} cell activation by soluble anti-CD3 plus APC (data not shown). Thus, splenic APC appear to augment CD45RB^{lo} activation when provided in trans to TCR cross-linking and inhibit activation when provided in cis to TCR cross-linking.

Cross-Linking CD4 Inhibits Proliferation of Memory but Not Naive CD4 T Cells Induced by Anti-CD3

The difference in activation of memory CD4 T cells by anti-CD3 seen with MHC class II^{+} versus MHC class II^{0} APC suggested that a negative signal was being transduced to memory CD4 T cells via interaction with MHC class II, whose only known receptor on these cells is CD4. Numerous studies have shown that proliferation and effector function of T cells can be inhibited by cross-linking CD4. However, the effect of CD4 cross-linking on the activation of normal naive and memory CD4 T cells has never been examined. To establish that a selective negative signal could be delivered through CD4 on the surface of a memory CD4 T cell, but not through CD4 on naive CD4 T cells, we sought to mimic the inhibitory effect of MHC class II^{+} APC by cross-linking CD4 with anti-CD4 MAbs in the presence of MHC class II^{0} APC. As shown in Figure 7A, proliferation of CD45RB^{lo} CD4 T cells to anti-CD3 in the presence of MHC class II^{0} APC was inhibited by addition of

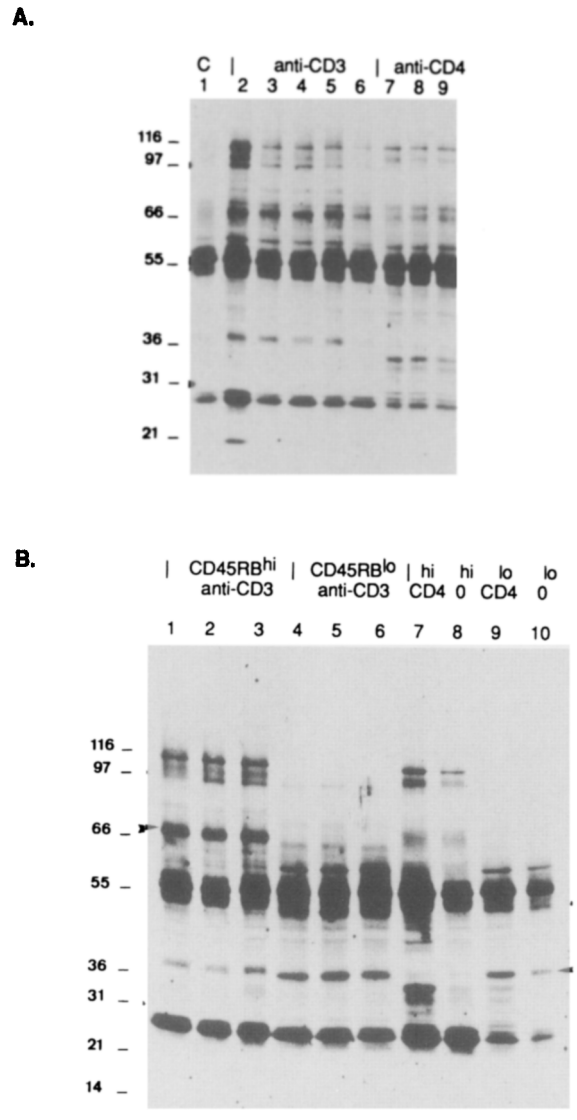

Figure 8. Comparison of Tyrosine-Phosphorylated Proteins in Resting, Anti-CD3- and Anti-CD4-Treated Whole CD4$^+$ Cells and CD4$^+$ CD45RB Isoform Subsets

(A) Kinetics of appearance of tyrosine-phosphorylated products in whole CD4$^+$ cells. Cells (1.6×10^6 equivalents per lane) were treated at 37°C for the times indicated with either anti-CD3 (C363.29B plus goat anti-rat IgG), or anti-CD4 (IgM RL172.5). Cell lysates were resolved on 5%–15% gradient SDS gels, transferred to nitrocellulose, resultant blots were hybridized with anti-phosphotyrosine antibody, and bands were revealed by chemiluminescence. Lane 1, untreated; lane 2, anti-CD3 for 30 s; lane 3, anti-CD3 for 2 min; lane 4, anti-CD3 for 5 min; lane 5, anti-CD3 for 10 min; lane 6, anti-CD3 for 30 min; lane 7, anti-CD4 for 30 s; lane 8, anti-CD4 for 2 min; lane 9, anti-CD4 for 5 min.

(B) Phospho-tyrosine blots of lysates from CD45RB^{lo} and CD45RB^{hi} cells. Each lane represents 1.5×10^6 cell equivalents. Lanes 1 and 4, anti-CD3, 10 min; lanes 2 and 5, anti-CD3 for 2 min; lanes 3 and 6, anti-CD3 for 30 s; lanes 7 and 9, anti-CD4 for 2 min; lanes 8 and 10, untreated controls. Molecular mass standards are designated (in kilodaltons): 116, 97, 66, 55, 36.5, 31, 21.5.

the anti-CD4 MAb GK1.5, but not with an isotype-matched control antibody. GK1.5-induced inhibition was apparent at low doses of antibody (data not shown). By contrast, addition of GK1.5 failed to inhibit the proliferative response of CD45RB^{hi} CD4 T cells to anti-CD3 together with either MHC class II^0 or MHC class II$^+$ APC (Figure 7B). These data are consistent with the results presented above, showing that only CD45RB^{lo} CD4 T cells are inhibited by contact with MHC class II$^+$ cells. Thus, our data demonstrate that MHC class II on APC can deliver a negative signal to memory but not naive CD4 T cells, and strongly suggest that this negative signal is delivered via CD4.

Memory and Naive Cells Display Different Patterns of Tyrosine Phosphorylation

The results presented above strongly suggest that signaling through both the TCR/CD3 complex and through CD4 in memory CD4 T cells differs from that in naive CD4 T cells. To determine whether differential activation via CD3 and differential inhibition via CD4 reflect distinct intracellular signaling in the two subsets, we examined the appearance of tyrosine-phosphorylated substrates in both whole CD4 T cells and CD45RB subsets immediately following CD3 or CD4 cross-linking. As shown in Figure 8A, resting whole CD4 cells display few tyrosine-phosphorylated bands (lane 1); following CD3 cross-linking for increasing times, major bands of 21 kDa, 42 kDa, 70–80 kDa, and 97–116 kDa appear (lanes 2–6). Following CD4 cross-linking by immunoglobulin M (IgM) anti-CD4, novel bands of 34 and 36 kDa appear and the 42 kDa and higher molecular mass bands seen with anti-CD3 stimulation lose their prominence (Figure 8, lanes 7–9).

A similar timecourse of stimulation of CD45RB^{lo} versus CD45RB^{hi} cells is compared in Figure 8B. In general, the patterns of tyrosine-phosphorylated substrates observed in resting, anti-CD3 cross-linked, and anti-CD4 cross-linked CD45RB subsets differs; fewer species of tyrosine-phosphorylated proteins appear in stimulated memory cells when compared with naive cells or whole CD4 cells. Nonactivated CD45RB^{lo} and CD45RB^{hi} cells express few tyrosine-phosphorylated substrates, with the exception of a 40 kDa band that is constitutively present in CD45RB^{lo} cells (Figure 8, lane 10) but not in CD45RB^{hi} cells (lane 8). After cross-linking CD4, the two bands of 34–36 kDa seen in whole CD4 cells are more strongly induced in CD45RB^{hi} cells (Figure 8, lane 7) than in CD45RB^{lo} CD4 T cells, which also exhibit a marked increase in phosphorylation of the 40 kDa band after CD4 cross-linking (lane 9). Thus, cross-linking CD4 on naive and memory CD4 T cells induces different patterns of tyrosine-phosphorylated substrates consistent with the distinct physiologic effects of CD4 ligation in these two populatons.

Cross-linking CD3 also produces different functional outcomes in naive and memory CD4 T cells and likewise generates strikingly distinct patterns of tyrosine-phosphorylated substrates. In response to anti-CD3 stimulation, strongly tyrosine-phosphorylated proteins of 42 kDa, 70–80 kDa, and three high molecular mass bands between 96–120 kDa appear in CD45RB^{hi} cells (Figure 8B, lanes 1–3), and likewise in whole CD4 cells (Figure 8A, lane 2), but are notably absent in CD45RB^{lo} cells (Figure 8B, lanes 4–6). The constitutive 40 kDa band in CD45RB^{lo} cells appears more strongly phosphorylated as a result of CD3 cross-linking (Figure 8B, lanes 4–6), and migrates faster

than the 42 kDa band observed in anti-CD3-treated CD45RB^{hi} cells. Only one high molecular mass band appears in anti-CD3-stimulated CD45RB^{lo} cells, corresponding to the lowest of the three high M_r bands seen in stimulated CD45RB^{hi} cells.

These data indicate that proximal signaling events transduced both through CD3 and CD4, namely the phosphorylation on tyrosines of various cytoplasmic substrates, differs strikingly between naive and memory cells.

Discussion

This study demonstrates both functional and biochemical differences in the activation of naive and memory CD4 T cells freshly isolated from normal donor mice. These two CD4 T cell subpopulations were defined by differential expression of the CD45RB isoform or isoforms detected by the MAb 16A, specific for the B exon of CD45 (Bottomly et al., 1989). We had previously shown that these subsets differ in their activation requirements, with memory CD4 T cells responding to anti-CD3 bound to plastic surfaces or cross-linked by Fc receptor–bearing fibroblasts, while naive CD4 T cells responded to anti-CD3 only in the presence of APC from T-depleted spleen. These earlier studies suggested that the activation requirements of naive CD4 T cells are more stringent than those of memory CD4 T cells (Luqman and Bottomly, 1992). This is consistent with results demonstrating that human memory cells (CD45RO$^+$) obtained from peripheral blood were more easily activated and more sensitive to stimulation through TCR/CD3 than naive cells (CD45RA$^+$) (Byrne et al., 1988; Horgan et al., 1990; Sanders et al., 1989).

However, in the present studies we noted the anomaly that memory CD4 T cells failed to respond to anti-CD3 presented by MHC class II$^+$ APC, whereas naive CD4 T cells were fully responsive to this stimulus. Although this result appeared paradoxical, other studies have demonstrated that memory CD4 T cells are less responsive than their naive counterparts to noncognate stimuli (Lee and Vitetta, 1992; Jenkins and Miller 1992). For example, when stimulated with staphylococcal enterotoxin B and APC, CD45RB^{lo} memory CD4 T cells failed to proliferate, whereas CD45RB^{hi} cells proliferated well (Lee and Vitetta, 1992).

CD4 T cell activation by anti-CD3 added in soluble form requires presentation by an FcR-expressing accessory cell. The present studies show that MHC class II molecules on FcR-bearing cells inhibit anti-CD3-induced proliferation and effector cytokine secretion by memory but not by naive CD4 T cells. This was established using MHC class II$^+$ and class II$^-$ FcγR$^+$ transfectants as well as T-depleted spleen cells from MHC class II–deficient mice. The likely target of the MHC class II molecules on CD4 T cells is CD4 itself (Doyle and Strominger, 1987), the only protein known to bind specifically to MHC class II, and indeed, ligation of CD4 by anti-CD4 antibody could fully reproduce the selective inhibition of memory but not naive CD4 T cells seen with anti-CD3 and MHC class II$^+$ APC. Previous studies with unseparated CD4 T cells demonstrating that CD4 cross-linking did not inhibit responses to anti-CD3 and APC (Newell et al., 1990) are not inconsistant with our results, because the inhibition of memory cells was likely masked by the lack of inhibition of naive cells.

There are several possible implications of our finding that CD4 ligation by MHC class II molecules inhibits stimulation of memory, but not naive, CD4 T cells. To begin with, such signals that inhibit cytokine release by memory or effector CD4 T cells may be important in limiting bystander effects. For example, as a memory CD4 T cell trafficks through lymph nodes, it will encounter many MHC class II–bearing cells that do not express its specific ligand, and some that do. Those that express specific antigen–MHC will activate the cell, causing it to secrete cytokines. A negative signal, which shuts off effector cytokine production, may be important in preventing these CD4 T cells from inappropriately activating adjacent B cells or macrophages that do not express antigen. Because memory CD4 T cells express higher levels of adhesion molecules than do naive cells (Gray, 1993), facilitating their interaction with APC, such selective negative signals delivered in the absence of antigen may also be necessary to prevent the persistence of irrelevant interactions between memory CD4 T cells and APC. This hypothesis (Tite et al., 1986) is supported by previous studies (Mazerolles et al., 1990) suggesting that antigen independent CD4–MHC class II interactions may promote CD4 T cell–APC separation. Moreover, we propose that activation by the appropriate MHC class II–peptide complex predominates over the inhibitory signal delivered by inappropriate contact with MHC class II–bearing APC, because MHC class II$^+$ APCs were unable to inhibit activation of CD45RB^{lo} cells by either FcR$^+$ MHC class II^0 APC or immobilized anti-CD3.

The finding of selective inhibition of memory cells via CD4 also has implications to the immune dysfunctions observed in AIDS. Memory CD4 T cell function is the first defect observable in HIV infection (Helbert et al., 1993), and it has been demonstrated that cross-linking of CD4 by gp120 causes apoptosis of T cells subsequently activated by TCR cross-linking with anti-CD3 (Moebius et al., 1992; Banda et al., 1992). Because memory CD4 T cells are shown here to be more susceptible than naive cells to negative signaling through CD4, it is intriguing to speculate that they may likewise be more susceptible to gp120-mediated apoptosis or inactivation.

Our results that memory CD4 T cells are selectively inactivated by MHC class II expression on APC appear in opposition to the fact that CD45RB^{lo} or memory cells respond well to antigen (Bottomly et al., 1989). We believe that the apparent discrepancy lies in the activating stimulus employed in these studies, anti-CD3 cross-linked by FcR bearing APC. Whereas antigen presented as peptides by MHC class II molecules forms a ligand that binds to both CD4 and the TCR promoting the physical association of these two molecules, which facilitates activation, stimulation by anti-CD3 involves a TCR/CD3–FcR interaction, which should not promote the TCR–CD4 interaction. Furthermore, we have observed that anti-CD3, and anti-CD3–Fab can dissociate TCR and CD4 on memory CD4 T cells and cloned T cell lines (J. Rojo, U. Dianzani, M. L., C. Janeway, and K. B., unpublished data). These data suggest that in contrast with antigen, anti-CD3 sterically inhib-

its the association of CD4 with the TCR. Finally, it has been shown (Dianzani et al, 1992) that IgM anti-CD4, which efficiently cross-links CD4, potently inhibits activation of a cloned CD4 T cell line by anti-TCR and anti-CD3 antibodies, but has little or no effect on the response of these same T cells to antigen. Thus, our experimental system has to be viewed as revealing inhibitory signals that are induced by cross-linking CD4 distinct from the TCR, which, we propose, constrain the activity of memory CD4 T cells but do not impede the proper functioning of these cells when they encounter specific antigen–MHC class II complexes on APC.

The selective susceptibility of memory CD4 T cells to negative signaling through CD4 and differential activation through CD3 strongly implies that both CD3 and CD4 are coupled to different biochemical signaling events in memory and naive T cells. We have shown here that following both CD3 and CD4 cross-linking, strikingly dissimilar patterns of tyrosine-phosphorylated proteins appear in the two CD45RB subsets. The pattern of tyrosine-phosphorylated proteins seen in CD45RB^{hi} parallels that seen with whole CD4 cells, indicating that the selecting antibody used to isolate the cells did not qualitatively affect signaling. There are more major tyrosine-phosphorylated proteins after CD3 cross-linking in naive cells than in memory cells, including major 70–80 kDa bands that are present exclusively in naive cells and may contain the ZAP-70 kinase that has been shown to be phosphorylated and activated immediately following TCR/CD3 stimulation (Chan et al, 1991). These data suggest that different kinases may be activated through TCR/CD3 in naive versus memory T cells and identification of those kinases may provide the key to determining the molecular basis of differential T cell activation. Although it has been shown in T cells clones that the p56^{lck} kinase can tranduce negative signals through CD4 (Haughn, et al., 1992), we have found no differences in CD4-associated and total p56^{lck} kinase activity between the two subsets by immunoprecipitation and in vitro kinase assay (data not shown), suggesting the involvement of other kinases in differential tyrosine phosphorylation after CD3 and CD4 ligation, and in preferential CD4-mediated negative signaling of CD45RB^{lo} cells.

It has recently been demonstrated in our laboratory that CD45 isoforms can influence activation through the TCR in CD45 isoform transfectants (Novak et al, 1994). We have previously shown by cocapping analysis that CD45 isoforms also modulate associations with CD4 and TCR/CD3, with the CD45 isoforms expressed by memory (CD45RB^{lo}) cells preferentially associating with CD4 and TCR/CD3 and the CD45 isoforms expressed by naive (CD45RB^{hi}) cells not associating with CD4 or TCR/CD3 (Dianzani et al, 1990). The physical context of TCR, CD4, and CD45 on CD45RB^{lo} and CD45RB^{hi} cell surfaces may thus direct the differential activation of both TCR- and CD4-associated tyrosine kinases, leading to the appearance of distinct species of tyrosine-phosphorylated substrates when CD4 or CD3 are cross-linked in the two subsets. Further evidence that CD45 isoform expression may govern the differential activation and CD4-mediated negative signaling observed in this study is suggested in studies using CD45 isoform transgenic mice. Thymocytes (normally CD45RB^{lo}) expressing a CD45ABC transgene (rendering them CD45RB^{hi}) became resistant to anti-CD4-mediated negative signaling (Chui et al, 1994), consistent with our results with peripheral CD45RB^{hi} cells. Interestingly, CD45RB^{hi} thymocytes from these ABC transgenic mice also exhibited major 70 and 80 kDa bands after anti-CD3 stimulation that were not present in stimulated CD45RB^{lo} thymocytes from null isoform transgenic mice (Chui et al., 1994), suggesting a biochemical correlation between CD45RB^{hi} isoform expression and the appearance of 70 kDa, 80 kDa, or both tyrosine-phosphorylated bands after activation.

In conclusion, our studies reveal that the function of CD4 expressed by memory T cells differs from that observed with naive T cells. The inhibitory actions are particularly important in CD4 T cells that express low molecular mass isoforms of CD45, perhaps because these associate physically with CD4 and modulate its activity. We also present here clear differences in signaling through CD3 and CD4 in the two CD4 subsets. Our findings have important implications for regulation of CD4 T cell effector function and for the defective action of memory CD4 T cells seen as the earliest effect of infection with HIV. Further biochemical characterization of intracellular events occurring in these two subsets following CD3 ligation, CD4 ligation, or both will enable us to determine the molecular mechanisms underlying the complex regulation of naive and memory CD4 T cell activation.

Experimental Procedures

Mice

Female BALB/c ByJ mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) and were maintained at the animal facility at Yale University (New Haven, Connecticut). All mice were used between 6–12 weeks of age. Class II knockout mice (RHAβo/o) originally derived from 129/Sv mice (Cosgrove et al., 1991) were provided by Dr. D. Mathis (Institut de Chimie Biologique, Strasbourg, France) and were bred and maintained at Yale University.

Antibodies

Unless otherwise indicated, all MAbs used were purified from supernatants from hybridomas maintained in this laboratory, using standard protein A or protein G affinity chromatography. The following MAbs were used in this study: C363.16A (rat IgG2a, anti-CD45RB) (Bottomly et al., 1989); C363.29B (rat IgG2c, anti-CD3ε) (Portoles et al., 1989); MEL-14 (rat IgG2a, anti-lymphocyte homing receptor) (Gallatin et al., 1983); IM7.8.1 (rat IgG2b, anti-Pgp-1) (Trowbridge et al., 1982); 2.4.G2 (rat IgG2b, anti-muFcγRII) (Unkeless, 1979); GK1.5 (rat IgG2b, anti-CD4) (Dialynas et al., 1983); anti-CD8 (rat IgG2a, clone 53-6.72) (Ledbetter and Herzenberg, 1979); anti-CD8 (rat IgG2b, clone 2.43), 212.A1 (mouse IgG2a, anti-I-A^{s}) (Landais et al., 1987); MKD6 (mouse IgG2a, anti-I-A^{d}) (Kappler et al., 1981); 11B11 (anti-IL-4) (Ohara and Paul, 1985); and XMG1.2 (anti-IFNγ) (Cherwinski et al., 1987).

Cell Lines and Transfections

The murine L cell lines DAP.3 and 44/14.B7, an I-A^{d}-expressing DAP.3 cell (König et al., 1992), were provided by Dr. R. N. Germain (National Institutes of Health, Bethesda, Maryland). These cells were cotransfected with expression vectors containing the full-length muFcγRIIb2 cDNA (Ravetch et al, 1986) constructed by cloning muFcγRIIb2 cDNA (provided by Dr. I. Mellman, Yale University), into a modified pSP72 vector containing the SRα promoter (Takebe et al., 1988) (unpublished construct provided by Dr. T. Novak, Yale University), along with pSV2hyg, encoding a hygromycin B resistance gene. Cells were transfected as monolayers using Lipofectin (GIBCO BRL, Gaithers-

burg, Maryland) according to the protocol of the manufacturer. Stable transfectants, designated L/FcR$^+$/II^0 and L/FcR$^+$/II$^+$, were selected in DMEM, 5% fetal calf serum, 10 mM HEPES, 2 mM glutamine, 50 U/ml penicillin–streptomycin sulfate, 300–350 μg/ml hygromycin B (Calbiochem, La Jolla, California) and 250 μg/ml G418 for L/FcR$^+$/II$^+$ cells. Resistant colonies were expanded and sorted twice for FcγRII expression on the FACStar (Becton-Dickinson, Mountain View, California) based on 2.4G2 staining.

Preparation of CD4 T Cells and Separation Into CD45RB Subsets

CD4 T cells were isolated by immunomagnetic selection from BALB/c splenocytes as described previously (Luqman and Bottomly, 1992). In brief, splenic CD4 T cells were isolated by negative selection using MAbs to CD8, and to class II, followed by incubation with anti-rat IgG-, anti-mouse IgG-, and anti-mouse IgM-coated magnetic beads (Collaborative Research, Bedford, Massachusetts); CD4 cells were collected by magnetic depletion and centrifuged through a 62%–85% percoll gradient to remove activated T cells and contaminating accessory cells. The resultant CD4 population, as assessed by staining with GK1.5, was 85%–90% pure. Separation of CD4 cells into CD45RB^{lo} and CD45RB^{hi} subsets, was carried out by MACS separation (Miltenyi et al., 1990) according to the protocol of the manufacturer (Miltenyi Biotec, Sunnyvale, California), with some modifications. In brief, CD4 T cells were incubated for 20 min on ice with C363.16A–biotin (anti-CD45RB), followed by incubation with strepavidin magnetic beads (Miltenyi Biotec, Sunnyvale, California). The stained CD4$^+$ T cells were applied to a MACS separation column fitted between a superMACS magnet. Nonadherent CD45RB^{lo} cells were collected first, columns were washed extensively, and bound CD45RB^{hi} cells were eluted outside the magnetic field and reapplied to the magnetic column two to three times to ensure thorough separation of CD45RB^{lo} and CD45RB^{hi} subsets. Purity of each population was assessed by staining with fluorescein isothiocyanate–avidin D (Vector Laboratories, Burlingame, California). The resultant CD45RB^{lo} population was >95% CD4, and the CD45RB^{hi} subset was 85%–90% CD4, as determined by GK1.5 staining.

Preparation of APCs

Splenic APCs were prepared by complement depletion of BALB/c or RHAβo/o splenocytes treated with anti-Thy1, anti-CD4, and anti-CD8 antibodies. Following complement depletion, cells were treated with 50 μg/ml mitomycin C (Boehringer Mannheim Biochemica, Federal Republic of Germany) as described previously (Luqman and Bottomly, 1992). Purity of the APC preparation was assessed by anti-class II staining for BALB/c, and anti-FcRII (2.4G2) staining for MHC class II^0 APC. Purity was >95% MHC class II$^+$ and/or FcRII$^+$ cells in both cases. In experiments where fibroblasts were used as APC, transfected fibroblasts were fixed in 1% paraformaldehyde at 3 × 10^6 cells/ml for 25 min at 25°C. Following extensive washing with phosphate-buffered saline, fixed cells were resuspended in media and incubated 4–6 hr at 37°C before using in assays.

Proliferation Assays

CD45RB^{lo} and CD45RB^{hi} CD4 T cells were cultured at 25,000 cells/well in 96-well flat-bottomed plates (Costar, Cambridge, Massachusetts) with soluble anti-CD3 in the presence or absence of anti-CD4, anti-FcR, or isotype-matched control antibodies at indicated concentrations and 75,000 splenic APCs/well or 50,000 fixed fibroblasts/well in Eagles high amino acid medium (Peck and Bach, 1973) supplemented with 5% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin sulfate, and 10 mM HEPES. To immobilize antibody on plates, anti-CD3 was diluted to indicated concentrations in 50 mM Tris–HCl (pH 9.5) and incubated 2–4 hr at 37°C. Wells were washed with medium twice before culturing 25,000 T cells/well. Cultures were pulsed after 4 days with 1 μCi [^{3}H]dTdR (6.7 Ci/mmol) per well and harvested 18–24 hr later on a Tomtec 96-well plate harvester. Radioactivity was quantitated by scintillation counting. Results are presented as averages of triplicates and standard errors were <20% unless otherwise indicated.

Cytokine Assays

IL-4 and IFNγ in supernatants were measured by bioassay. For IL-4 measurement, serial dilutions of supernatant from cultures described above were incubated were added to 10,000 CT.4S indicator cells (Hu-Li et al., 1989) in the presence or absence of anti-IL-4 antibody. Cultures were pulsed after 48 hr with 1 μCi [^{3}H]dTdR and harvested 18–24 hr later. Standard plates were set up with serial titrations of recombinant mouse IL-4 (Becton-Dickinson, Bedford, Massachusetts) and units were calculated from standard curves with 1 U being defined as the amount of IL-4 that gave half-maximal proliferation. In all assays, CT.4S proliferation was completely inhibited with anti-IL-4. For quantitation of IFNγ, dilutions of supernatant were incubated with 5000 WEHI-279 cells (Warner et al., 1979) in the presence or absence of anti-IFNγ antibody. Proliferation was assessed after 48 hr, with 1 U of IFNγ defined as the concentration that gave half-maximal inhibition of WEHI-279 proliferation calculated from the standard curve obtained with mouse IFNγ standard (Genzyme Corporation, Cambridge, Massachusetts). In all assays, inhibition of proliferation was blocked by anti-IFNγ.

Phosphotyrosine Immunoblots

CD4, CD45RB^{lo}, and CD45RB^{hi} cells were isolated as described above and incubated in media overnight at 37°C. This step was necessary to eliminate the high backround of tyrosine-phophorylated proteins that occurs after prolonged incubation at 4°C (data not shown). CD45RB^{lo} and CD45RB^{hi} cells were resuspended in RPMI without fetal calf serum at 3 × 10^7 cells/ml. For CD3 cross-linking, 1.5 × 10^6 cells in 50 μl RPMI were incubated for 15 min on ice with C363.29B antibody, cells were pelleted and resuspended in 50 μl RPMI, and then 2 μg goat anti-rat F(ab')$_2$ were added and cells were placed for varying amounts of time at 37°C. For CD4 cross-linking, RL172.5 (IgM anti-CD4) was added to cells on ice, and incubated for 30 s to 2 min at 37°C. Cells were lysed in lysis buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Pefabloc-sc, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate), kept on ice for 30 min, and centrifuged at 13,000 rpm at 4°C to remove nuclear material. Proteins in the lysates were resolved on 5%–15% SDS gradient gels, transferred electrophoretically to nitrocellulose, and hybridized to anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology Associates, Lake Placid, New York) followed by horseradish peroxidase–coupled goat anti-mouse IgG (Biorad, Hercules, California). Bands were revealed by chemiluminescence using the enhanced chemiluminescence Western blotting detection reagents (Amersham) according to the protocol of the manufacturer.

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